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Original Article

COMPARISON STUDY OF ANTIOXIDANT, ANTIMICROBIAL AND CYTOTOXIC PROPERTIES OF SECONDARY METABOLITE AND PROTEIN EXTRACTS FROM *CLINACANTHUS NUTANS*

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ABSTRACT

Objective: The present study aims to evaluate the antioxidant, antimicrobial and cytotoxic properties of the secondary metabolites and proteins extracted from *Clinacanthus nutans*.

Methods: Methanol (ME) and acetone (AC) and also protein (PE) extracts were obtained from *Clinacanthus nutans*. Antioxidant activity of sample extracts was examined by using 1,1-Diphenyl-2-Picrydyl Hydrazyl (DPPH) assay and antimicrobial activity was examined by using the agar diffusion method against *Bacillus subtilis*, *Bacillus cereus, Streptococcus pyrogenes, Escherichia coli*, and *Pseudomonas aeroginosa* bacterial strain. The cytotoxic potential was determined by brine shrimp lethality assay.

Results: Methanol extract (ME) demonstrated to have the highest antioxidant activity with IC50 value of 98.84 µg/ml, followed by acetone extract (AE) with IC50 value of 134.83µg/ml, and protein extract (PE) with IC50 value of 353.49 µg/ml. Among all of the sample extracts, only methanol crude extracts (ME) displayed moderate inhibition against Gram-positive *B. cereus* (7.33±1.15 mm) and *S. pyrogenes* (8.67±0.57 mm) at concentration of 100 mg/ml, while both acetone (AE) and protein (PE) extracts had no activity against all tested microorganisms. All extracts from methanol (ME), acetone (AE) and protein (PE) exhibited significant cytotoxic activity against brine shrimps at LC_{50} 7.2 µg/ml, 1.42 µg/ml and 70.6 µg/ml respectively.

Conclusion: These data proved that methanol extract from the leaves of *Clinacanthus nutans* is the most potent among all samples tested and has the potential to be developed as an antioxidant, antimicrobial and cytotoxic agents. Whilst, acetone and protein crude extracts have potent antioxidant and cytotoxicity activities.

Keywords: *Clinacanthus nutans,* Antioxidant, Antimicrobial, Cytotoxicity, Methanol extract, Acetone extract, Protein extract

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INTRODUCTION

Plants possess biologically active compounds that are adopted in traditional medicine for thousands of years. Over the last decades, research on medicinal plants has aroused great interest as there were many reports on adverse effects using synthetic drugs. Thus, bioactive compounds derived from plants remain a significant route for the development of novel therapeutic agents to treat diseases, like cancer, cardiovascular, metabolic and degenerative diseases.

Clinacanthus nutans, (Burm. f.), locally known as Sabah snake grass, belongs to the family of *Acanthaceae*. It is found as a small shrub in South East Asian regions, which can grow up to 1-3 meters tall [1]. Traditionally, the extract from the leaves has been used to treat inflammatory conditions, particularly insect bites, skin rashes, infections of varicella-zoster virus (VZV) and herpes simplex virus (HSV) [2, 3]. The consumption of this plant in the form of herbal tea has also been suggested to treat diabetes mellitus, fever, diarrhoea and dysuria [4].

Apart from the traditional usage of *C. nutans*, a considerable amount of studies has been conducted on *C. nutans* to elucidate the protective activity such as antiviral [5, 6], anti-inflammatory [7], anti-venom [8] and immune response by modulating lymphocyte proliferation [9]. Many studies have been reported that *C. nutans* possess antimicrobial activity [10-13]. The leave extracts have also been reported to exhibit strong antioxidant properties [14]. *In vitro* studies suggested that their potent antioxidant attribute could be correlated to anticarcinogenic activity. Besides, Yong *et al.*, 2013 have found that chloroform extracts from C. *nutans* exert the strongest chemoprotective effects against some cancer cell lines such as K-562 and Raji cells and could claim as a therapeutic target to reduce the incidence of cancer [13, 15].

However, there are contradictive shreds of evidence on using different solvent for extracting the therapeutic bioactivities compounds from *C. nutans* [15-17]. In addition, protein extract from the leaves of *Clinacanthus nutans* has not been studied before. Hence, in this study, methanol, acetone, as well as protein extracts of *Clinacanthus nutan's* leaves are investigated to study for their differential antioxidant, antimicrobial and cytotoxic activities.

MATERIALS AND METHODS

Preparation of plant material

The whole plant of *Clinacanthus nutans* was collected from Kota Kinabalu, Sabah, Malaysia (5.9804 °N, 116.0735 °E); the leaves and stems were separated, air-dried at room temperature for 6–7 d and ground to a powder before extraction.

Extraction of secondary metabolites

The extraction yield was calculated to reflect the degree of the solvent's efficacy to extract compounds from the plant and also percentage of extract recovered in dry mass compared to the initial mass of plant [18]. The powdered dried leaves (35g) were macerated in 350 ml of methanol and acetone separately for a day at room temperature (27 °C) with occasional shaking. The extract was filtered and concentrated under vacuum at 40 °C using a rotary evaporator to produce the crude extract. Freeze-dried extract was then stored at 4 °C in a sealed bottle until further analysis.

Preparation of protein extract

100 g of fresh *Clinacanthus nutans* leaves were blended with 1000 ml of extraction buffer for 10 min using a blender and the protein content was extracted overnight at 4 °C in the extraction buffer (8.01

g/l NaCl, 0.20 g/l KCl, 1.78 g/l Na2HPo4, 0.27 g/l KH_2PO_4). After filtration through Whatman 1 paper, the crude protein extract of *Clinacanthus nutans* leaves was centrifuged at 15,000 rpm for 20 min. The supernatant was then collected and ammonium sulphate was added to a final concentration of 60 % saturation and left overnight at 4 °C. After centrifugation at 15 000 rpm for 30 min, the precipitate was dissolved in deionized water and then dialysed against distilled water for 24 h at 4 °C. The final *Clinacanthus nutans* crude protein extract was collected and freeze-dried before kept at 4 °C for further use [19].

Bradford protein quantification assay

Bovine Serum Albumin (BSA) was used as a standard. BSA stock solution of 100 µg/ml was prepared. Then dilution was done in order to get the BSA solutions with concentrations of (10 µg/ml, 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml, 100 µg/ml) in a total volume of 100 µl. After that, 1 ml of Bradford reagent was added to each test tube containing the different concentrations of BSA solutions. At the same time, 100µg/ml of *Clinacanthus nutans* crude protein extract was prepared by dilution and added with 1 ml of Bradford reagent. All these test tubes were incubated for 5 min at room temperature and the absorbance at 595 nm was recorded against a reagent blank (100 µl buffer+1 ml Bradford reagent) by using a spectrophotometer. Then, a standard curve of Absorbance (595 nm) versus Concentration (µg/ml) of BSA was plotted. The unknown concentration of protein in (µg/ml) in the crude protein extracted from the leaves of *Clinacanthus nutans* was determined from the standard curve.

DPPH free radical scavenging assay

Free radical scavenging activity was determined using 1,1-diphenyl-2-picrylhydrazyl, DPPH (Sigma-Aldrich, USA) spectro-photometrically. Here, 0.1 ml of sample extracts (0.625 to 5 mg/ml) were mixed with 3.9 ml of 0.004% DPPH solution in methanol. The final volume of the solution was adjusted with methanol to 4 ml. The mixture was shaken vigorously and incubated in the dark area at room temperature for 30 min. The scavenging activity was observed by the discolouration of DPPH solution from purple to yellow. The absorbance of the sample solution was measured using a spectrophotometer (Thermo Scientific) at 517 nm using methanol as blank and the results were recorded. Ascorbic acid was used as positive control and reference antioxidant. All measurement was taken in triplicate. The DPPH radical scavenging activity was calculated using the following formula:

$$\% \text{ radical scavenging} = \frac{(Ac - Acb) - (As - Asb)}{(Ac - Acb)} \text{ x } 100$$

Ac: the absorbance of water and DPPH in methanol

Acb: the absorbance of water and methanol without DPPH

As: the absorbance of sample and DPPH in methanol

Asb: the absorbance of sample and methanol without DPPH

Antibacterial activity assay

Disc diffusion method was employed to test the antibacterial activity. Briefly, bacterial strains were grown in 100 ml of sterile nutrient broth and incubated at 37 °C overnight. Petri plates containing 20 ml of Muller Hinton agar was prepared for this assay. About 100 µl of the bacterial culture (10^6CFU) was spread on the surface of the agar and allowed to dry for 5-10 min. Plant extracts with the final concentration of 10, 25, 50 and 100 mg/ml were prepared by dissolving in dimethyl sulphoxide (DMSO). The Whatman Grade AA filter paper discs (4 mm in diameter) impregnated with different concentrations of crude extracts were placed on the agar under aseptic conditions. The plates were incubated overnight in an upright position at 37 °C. After 24 h, the antibacterial activity was determined by measuring the diameter of the inhibition zone around the paper discs in millimeters. The tests were performed in triplicate for each extract against the test microorganisms. The microorganisms were obtained from Biochemical Lab, Faculty of Science and Natural Resources, Universiti Malaysia Sabah, Kota Kinabalu, Sabah.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration was determined by the disc diffusion method. MIC was determined by the highest dilution of the crude extracts that inhibited bacterial growth. The minimum sample concentration that inhibited the growth of bacteria after 24 h of incubation at 37 °C was recorded as MIC.

***Artemia salina* (brine shrimp) lethality assay**

About one gram of dried cysts of brine shrimp was hatched in artificial marine water (1.2-3.0% NaCl in distilled water). The water was kept in mild motion by aeration at 27 °C for 48 h. Each sample was prepared as 10, 100 and 1000µg in 1% DMSO respectively. 0.5 ml sample extract was added to a series of test tubes and 10 nauplii from the hatching beaker were transferred into the test tubes with the final volume of 5 ml containing an aliquot of seawater each. 1% mercury chloride was used as positive control and distilled water was used as the negative control. Tests were done in triplicate. The test tubes were then placed in the dark at room temperature. After 24 h incubation, the number of dead nauplii was counted to get percentages of mortality (% M) using the following formula:

$$\text{percentage of mortality} = \frac{\text{Total nauplii} - \text{Live nauplii}}{\text{Total nauplii}} \text{ x } 100$$

Nauplii found at the bottom of test tubes were considered dead.

Statistical analysis

Results were expressed as mean±standard deviation (SD, n = 3) and analysed by one way ANOVA using SPSS software (SPSS 21). $P<0.05$ was considered a statistically significant difference when compared to the respective control group.

RESULTS AND DISCUSSION

Extraction of crude secondary metabolites

The methanol extract (ME) demonstrated to have a higher percentage of yield (12.42 % w/w; 4.35 g) in comparison to acetone extract (AE) with 10.91% w/w; 3.82 g of net yield. The results obtained are consistent with previous studies, which reported that methanol is an effective solvent in extracting and recovering polyphenols as compared to other solvents with different degree of polarity [20]. This data was also in agreement with the study done by Jaiwal and co-workers who indicated that among the solvents tested, methanol was found to be more efficient than acetone for extraction [21] of polyphenol compounds. In addition, El-Chaghaby and colleagues reported that aqueous mixtures of methanol and acetone are recommended as effective solvents for secondary metabolites extraction [22].

Extraction of protein and quantitative determination of crude protein concentration

Protein extracts (PE), on the other hand, had much lower yield percentage as compared to secondary metabolite methanol and acetone extracts; from the 100g of fresh *Clinacanthus nutans* leaves, there was only 0.35g (0.35% yield) of protein extract obtained. Quantitative Bradford assay showed that the concentration of protein crude extract is 12.16 µg/ml. This is consistent with other studies that showed that protein extract from plants are of the lowest yield as compared to secondary metabolites [23].

DPPH radical scavenging activity

The antioxidant activities of *C. nutans* extracts were determined using DPPH radical scavenging assay, which is based on the reduction of DPPH by sample and the discoloration from purple to yellow with the absorption at 517 nm. The percentage of absorbance denoted as IC_{50} value (µg/ml). Low IC_{50} value indicated that the sample has significant antioxidant activity. In the present work, all sample extracts (ME, AE, PE) from *C. nutans* were found to have antioxidant property in a dose-dependent manner as shown in table. 1. Ascorbic acid was used as the standard antioxidant. Antioxidant activity of all the tested samples is in the order of ME>AE>PE, and their antioxidant activities were significantly different from statistical analysis ($P<0.05$).

The high antioxidant activity of ME is consistent with its low IC_{50} value of 98.84µg/ml; while AE showed IC_{50} value of 134.83µg/ml and PE has the highest IC_{50} value of 353.49µg/ml. Table 1 shows the IC_{50} values of methanol (ME), acetone (AE) and proteins (PE) extracts of *C. nutans* in comparison to the standard ascorbic acid. *C. nutans* may contain flavonoids; a class of secondary plant phenolic, which can be easily extracted with polar solvents such as methanol and possess significant antioxidant and chelating properties [20, 24]. Previous studies also suggested that the presence of phenolic compounds in the extract highly correlates with the DPPH radical scavenging activity [20]. Though acetone is suggested to be one of the good solvents for extracting non-polar compounds, namely terpenoids. However, chemical compounds that exert strong antioxidant activity such as glycosides, saponins, flavonoids, alkaloids cannot be entirely extracted using acetone alone because of its limited ability to extract polar compounds. Therefore, this might be the reason why the acetone extracts exerted lower antioxidant activity as compared to methanol extracts in the present study. Nonetheless, both methanol and acetone extracts of *Clinacanthus nutans* have exhibited a significant amount of radical scavenging activity although the scavenging activity of acetone extracts is relatively lower than methanol extracts.

Though protein extract (PE) of *Clincanthus nutans* exhibited the lowest radical scavenging activity among all samples extracts with the IC_{50} value of (353.49µg/ml), but the fact that PE has the IC_{50} value in the range of µg/ml is regarded to be relatively strong antioxidant activity considering the low protein recovery percentage and of a crude protein extract. According to Ryan *et al.*, (2008), proteins have excellent potential as an antioxidant because they can inhibit lipid oxidation through multiple pathways, including the inactivation of reactive oxygen species, scavenging free radicals, chelation of prooxidative transition metals, reduction of hydroperoxides, and alteration of the physical properties of food systems [25]. Hence, this finding can be fundamental in the identification of potent antioxidant agents attributed by the capacity of peptides from *Clincanthus nutans* interacting with free radicals [26].

Table 1: IC_{50} (µg/ml) values of sample extracts from *Clinacanthus nutans* as compared to ascorbic acid as a standard antioxidant in DPPH free radical scavenging activity

Sample extract	IC_{50} (µg/ml)
Methanol (ME)	98.84
Acetone (AE)	134.83
Protein (PE)	353.49
Ascorbic acid	10.25

Antibacterial activity

The degree of antimicrobial activity of plant extracts is associated with their zone of inhibition against test microorganisms. In this study, the antimicrobial activity of different extract of *C. nutans* was evaluated by disk diffusion method. The bacteria chosen were Gram-positive (*Bacillus subtilis, Bacillus cereus, Streptococcus pyrogenes)* and Gram-negative (*Pseudomonas aeruginosa* and *Escherichia coli*). These test organisms were chosen in this study because they give rise to spectre of bacterial infections and also these microorganisms have reported being increasingly resistant to conventional antibiotics. The results of the antibacterial activity of ME, AE and PE of *C. nutans* are presented in table 2. The methanolic extract (ME) of *C. nutans* exhibited moderate inhibition against Gram-positive *B. cereus* (7.33±1.15 mm) and *S. pyrogenes* (8.67±0.57 mm) at a concentration of 100 mg/ml, with MIC value of 100 mg/ml for both *B. cereus* and *S. pyrogenes.* Whereas AE and PE were found to have no antimicrobial activity against all tested microorganisms.

The present observation indicating that kanamycin (positive control) produced the largest zone of inhibition against all tested bacterial strains as compared to the sample extracts; this may be due to the fact that the plant sample extracts are of crude form, which contains a relatively lower concentration of bioactive compounds [27].

Our results confirmed that methanolic extract of *Clinacanthus nutans* had antimicrobial activity against *B. cereus* and *S. pyrogenes*, denoting that the active antimicrobial compounds can be better extracted with methanol as compared to other solvents. It was revealed that the methanolic extract of *Clinacanthus nutans* contains phytochemicals such as saponins, phenolic compounds, flavonoids, diterpenes and phytosterols, of which most of these compounds were recorded to possess antibacterial activity [15].

Cytotoxicity of brine shrimp lethality assay

The cytotoxicity effect of sample extracts was done using brine shrimp lethality assay and exposing sample extracts (ME, AE, PE) to *Artemia salina* for 24 h. Mercury chloride was used as a positive control, as described by Meyer *et al.,* 1982 [28].

Results obtained are shown in table 3. The higher the cytotoxicity of the extracts, the higher the lethality percentage of the brine shrimps.

Table 2: Antimicrobial inhibition zone of sample extracts using disc-diffusion method

Test microorganism	Antimicrobial inhibition zone (mm)			
	Kanamycin	ME	AE	PE
B. subtilis (G+)	25.66±0.57	NIL	NIL	NIL
B. cereus (G+)	28.66±1.15	7.33±1.15	NIL	NIL
S. pyrogenes (G+)	27.33±0.57	8.67±0.57	NIL	NIL
E. coli (G-)	27±1.73	NIL	NIL	NIL
P. aeruginosa (G-)	25.66±0.57	NIL	NIL	NIL

NIL =No zone of inhibition was observed. Values are mean±SD for three replicates (n=3). Kanamycin was used as a positive control

Table 3: Percentage of Lethality of Brine shrimps against methanol, acetone and protein extracts from *Clinacanthus nutans*

	Percentage of lethality (%)			
Conc. (µg/ml)	ME	AE	PE	MC
25	50.00±20.00	63.33±5.77	0.00±0.00	76.67±5.77
50	80.00±10.00	83.33±15.27	0.00±0.00	96.67±5.77
100	93.33±11.54	100±0.00	0.00±0.00	100.00±0.00
500	100.00±0.00	100±0.00	0.00±0.00	100.00±0.00
1000	100.00±0.00	100±0.00	3.33±5.77	100.00±0.00

Note: ME-Methanol Extract; AE-Acetone extract; PE-Protein Extract; MC-mercury Chloride. Data shown are the means±SD of three replicates.

From the result obtained, the percentage of lethality (%) of the brine shrimps increased as the sample concentration increased. The trend of lethality effect among all sample extracts is in the order of PE<ME<AE, whereby AE (acetone extract) exhibited to have the highest cytotoxicity followed by ME (methanol extract) and PE (protein extract). Based on the SPSS analysis of one way ANOVA significance analysis, there is a significant difference ($p<0.05$) between ME, AE, PE and also mercury chloride (ME). It is interesting to note that at the tested concentration of 1000 µg/ml, protein extract (PE) exhibited only a minimal (3.33±5.77) % of lethality while both methanol (ME) and acetone extract (AE) demonstrated (100.00±0.00)% of lethality. The cytotoxicity effect of ME and AE is apparently comparable to the level of lethality of positive control mercury chloride (MC) in which 100% lethality of ME and AE was recorded at 500 µg/ml and 100 µg/ml, respectively.

The data of LC_{50} (µg/ml) value is shown in table 4. AE exhibited the lowest LC_{50} (µg/ml) at 1.42 µg/ml, followed by ME at 7.20 µg/ml and PE recorded to have the highest LC_{50} (µg/ml) value at 70.6 µg/ml, implicating the cytotoxicity trend of the sample extracts is in the order of AE>ME>PE.

The brine shrimp lethality bioassay has been used extensively in the primary screening of the crude extracts and it has been established that the cytotoxic compounds that exhibit significant activity in this assay provide a promising indication of possible cytotoxic properties [28]. Hence, this assay can be recommended as a guide for the detection of antitumour and pesticidal compounds because of its simplicity and low cost [29]. The brine shrimp results are interpreted and categorized based on its LC_{50} value. The LC_{50} value less than 1.0 µg/ml is regarded as highly toxic, LC_{50} 1.0-10.0 µg/ml as toxic, LC_{50} 10.0-30.0 µg/ml as moderately toxic, LC_{50} more than 30 and less than 100 µg/ml as mildly toxic and finally, LC_{50} value more than 100µg/ml as non-toxic [30]. As the previous literature reported that LC_{50} value below 250 µg/ml has a likelihood of yielding anticancer compounds, thus all AE, ME and PE from *C. nutans* could be considered as a promising source of cytotoxic compounds for cancer treatment [31-34].

Table 4: LC_{50} value of methanol and acetone extracts against the brine shrimp, Artemia salina

Plant extract	Brine shrimp LC50 (µg/ml)
Methanol extract	7.20
Acetone extract	1.42
Protein extract	70.6
Mercury chloride (Positive control)	0.57

CONCLUSION

The study proved that methanolic extract from the leaves of *Clinacanthus nutans* is the most potent extract among all samples, which contains a considerable amount of bioactive compounds that attributed towards its antioxidant, strong cytotoxicity and antibacterial activities against Gram-positive *B. cereus* and *S. pyrogenes*. On the other hand, acetone and protein extracts exhibited antioxidant, strong cytotoxicity activity but no antibacterial activity against all microorganisms tested. Nonetheless, it is imperative to note that all the methanol, acetone and protein extracts demonstrated strong cytotoxicity activity, which stipulated *Clinacanthus nutans* is a potent candidate for an anti-cancer agent. Further investigation should be carried out to assess the chemical constituent responsible for its strong cytotoxic and antioxidant activities.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declare none

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